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Short Communication

Producing the sulfur-containing metabolite asparaptine in *Asparagus* calluses and a suspension cell line

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Abstract Asparaptine, a conjugate of L-arginine and asparagusic acid, was found in green asparagus (*Asparagus officinalis*) using ultrahigh-resolution metabolomics for sulfur-containing metabolites (S-metabolites), called S-omics. Asparaptine has been shown to inhibit the activity of angiotensin-converting enzyme. Larger amounts of this S-metabolite are therefore required for further analysis; however, there are limitations that asparagus is a perennial plant and its spears, wherein asparaptine accumulates, can be mainly harvested at the spring to summer season. In order to overcome these, we prepared a callus and suspension cell line from green asparagus. Untargeted metabolome analysis using liquid chromatography-tandem mass spectrometry was performed in the materials as well as spears and three calluses derived from wild type *Asparagus*. The analysis demonstrated that the amount of asparaptine in the callus derived from the green asparagus was more than the others per mg dry weight. The suspension cell line treated with methyljasmonate showed the induction of asparaptine, suggesting that the asparaptine production is modifiable under appropriate culture conditions. The described materials can be utilized for the production of asparaptine and in integrated metabolomics to study the biosynthesis of this S-metabolite, which is currently unknown.

Key words: asparagus, asparaptine, callus, metabolomics, specialized metabolite.

Asparagus (*Asparagus officinalis*) is one of the world's most important staple vegetables. Its edible spears are mainly harvested in the spring to summer season. Interestingly, asparagus spears accumulate unique specialized (previously called secondary) metabolites including flavonoids, saponins, and sulfur (S)-containing metabolites (S-metabolites) such as asparaptine, a conjugate of L-arginine and asparagusic acid (Figure 1). Asparaptine was found from green asparagus using metabolomics of S-metabolites, S-omics, by ultrahigh-resolution mass spectrometry (MS) (Nakabayashi et al. 2013). Generally, screening of particular metabolites requires the use of common structural feature(s) in MS-based metabolomics. Due to the extensive chemical diversity in S-metabolites, it has been difficult to identify such feature(s). Recently, however, the S-omics has enabled us to utilize the ^{34}S isotopic ion as the common feature (Nakabayashi and Saito 2017). By using the differences between the monoisotopic ion and the ^{34}S isotopic ion on the exact mass and signal intensity, the monoisotopic ion of asparaptine was characterized from the metabolome data. Since asparaptine was a new metabolite in asparagus, no previous structural information existed in the available databases. Therefore, the S-metabolite was purified from a large amount of asparagus and its structure was elucidated through nuclear magnetic resonance analyses, liquid chromatography-tandem mass spectrometry (LC-MS/MS), and amino acid analysis. Significantly, this metabolite showed inhibitory activity against angiotensin-converting enzyme (ACE), which is an important regulator of the blood pressure mechanism in animals. Hence, it is concluded that the metabolite is a natural ACE inhibitor found in asparagus (Nakabayashi et al. 2015). Furthermore, the metabolite is suggested to be effective for lowering blood pressure in animals, including humans. Larger amounts of asparaptine are required for further analyses; however, harvesting asparagus spears is inadvisable due to its high associated costs and inefficiency. In addition, since asparagus is a perennial plant, obtaining it sustainably may take years. Therefore, materials such as callus and suspension cell, which can be used for large-scale culture, are essential.

A callus and suspension cell line were derived from

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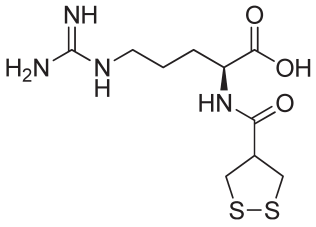

Figure 1. Structure of asparaptine.

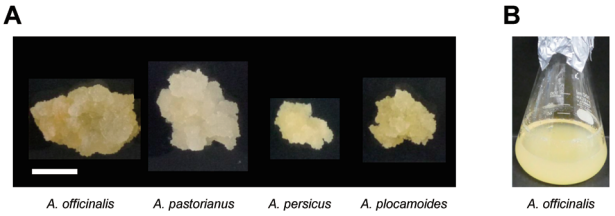

Figure 2. Asparagus materials used in this study. (A) Calluses derived from *A. officinalis* (green asparagus), *A. pastorianus*, *A. persicus*, and *A. plocamoides* as wild type. Bar indicates one cm. (B) Cultured cells derived from *A. officinalis*.

green asparagus spears in the Medicinal Plant Garden at Hoshi University (Figure 2A, 2B). In comparison with calluses derived from the wild type asparagus genus plants, the growth rate of the callus from the green asparagus was relatively faster under the medium utilized in this study (Figure 2A). The harvested samples were freeze-dried prior to metabolome analysis. With a view to evaluating asparaptine, we performed untargeted analysis using LC-MS/MS in the calluses, suspension cell line, and the freeze-dried edible green asparagus spear sample. Metabolome data including MS and MS/MS spectra were comprehensively acquired by data-dependent analysis in the positive ion mode. The signal intensity of the MS spectra was divided using that of the internal standard lidocaine. The total number of ions in the MS/MS was 641. The relative signal intensity of the ions was transformed to $\log_2$. Finally, the *z*-score was calculated using the $\log_2$-transformed value. Chemical assignment was performed using the existing metabolite library. Metabolite information including molecular formula and exact mass was extracted from the Dictionary of Natural products 28.1 using the word "asparagus", and the exact masses were then calculated for chemical assignment. The assignment was performed within 10 mDa mass error against each exact mass. Lastly, the metabolite information was assigned to 53 ions (Figure 3 and Supplementary Table 1). Of these, the ions derived from rutin, protodioscin, and asparaptine were identified using the authentic standard compounds.

Rutin is a flavonol glycoside, which has showed antioxidative activity (Makris and Rossiter 2001). Recently, the localization of rutin in asparagus was identified using a multi metabolomics approach involving LC-MS/MS and imaging mass spectrometry (Nakabayashi et al. 2019). The study clearly demonstrated that rutin accumulates in the epidermis of the asparagus spears. Furthermore, it was determined that this flavonol is also found in the developmental tissues and protoxylem. In the current analysis, rutin was detected in the spears; however, it was not identified in the calluses and suspension cell line due to the dark conditions used in the study.

Interestingly, saponins were assigned to detected ions using the existing metabolite library (Figure 3A). Protodioscin, a furostan type of saponins, was detected in the spears and suspension cell line. Moreover, spirostan type of saponins were assigned in the calluses and cultured cells (Supplementary Table 1). In order to identify these metabolites, isolation and structure elucidation must be performed because no commercially authentic standards are currently available.

As expected, asparaptine was detected in the callus and suspension cell line derived from the green asparagus. The amount of asparaptine was quantified in all samples (Figure 3B). Remarkably, the amount of asparaptine in the callus from the green asparagus was around twice than that in the asparagus spears, suggesting the callus as a potential candidate for large scale culture. On the other hand, the suspension cell line produced a smaller amount of asparaptine. However, methyljasmonate (MeJA) stimulated the secretion of asparaptine from the cells into the liquid medium. This resulted in approximately ten times more than the control samples. Other phytohormones, including gibberellin A_3 (GA_3), and indole acetic acid (IAA), also induced the secretion of asparaptine into the medium (Figure 3C). The relative signal intensity of asparaptine remained approximately the same in the cultured cells following the phytohormone treatment. This suggests that the secretion can be regulated to produce more asparaptine using appropriate chemicals and conditions.

Since asparagus is a perennial plant, it generally takes nearly three years for asparagus spears grown from seeds to be ready for harvest. Using the callus or suspension cell line derived from the green asparagus to obtain asparaptine is, therefore, time-efficient in large-scale culture. Furthermore, asparaptine can be obtained from the cultured materials and the medium. Synthetic biology approaches using genes involved in the biosynthetic and exported mechanisms have the potential to be utilized to increase the production of asparaptine. Unfortunately, all the mechanisms for asparaptine are largely unknown. The described materials can be used for transcriptome analysis using next generation sequencer to identify the key genes. A comparative analysis, using the materials treated with MeJA, will assist in narrowing down the candidate genes.

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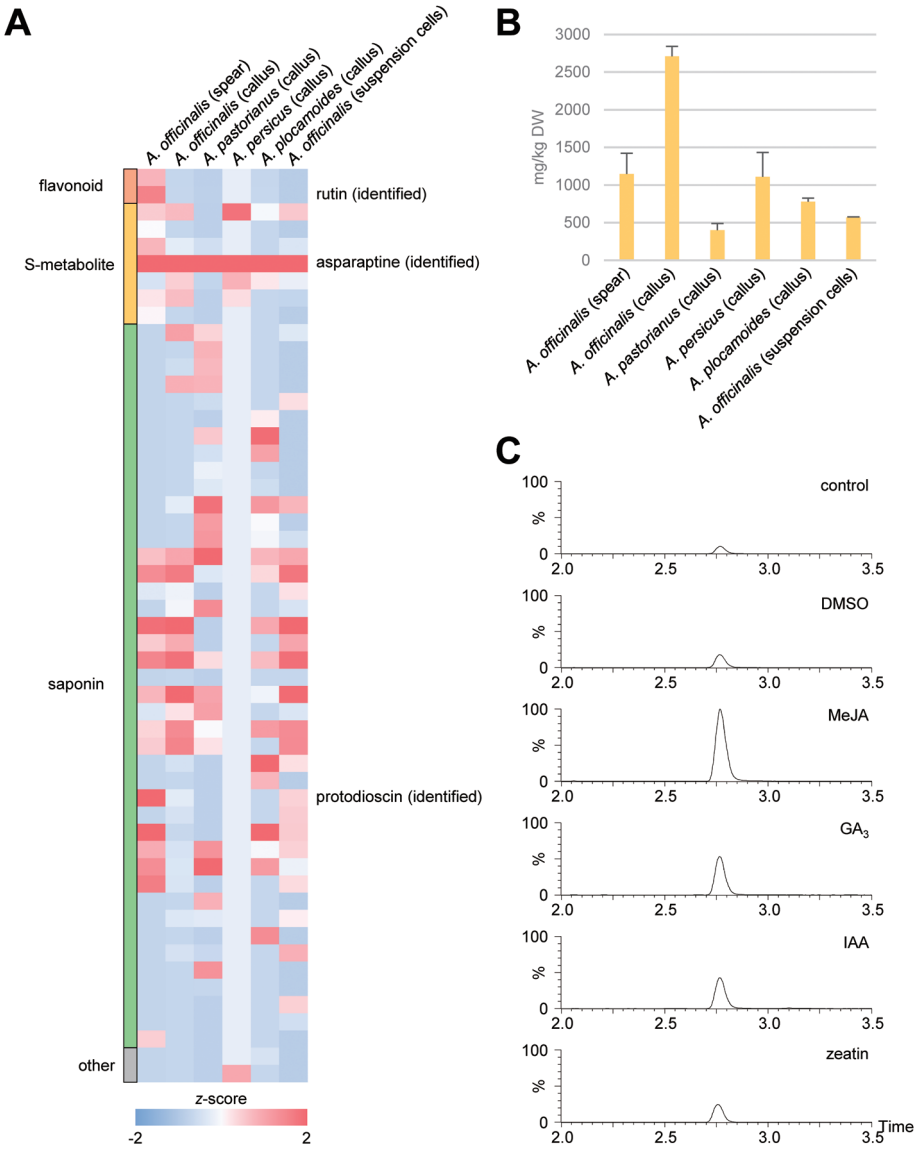

Figure 3. Profiling specialized metabolites including asparaptine in the calluses and susppnsion cell line. (A) Heat map of specialized metabolites assigned using the existing metabolite library. The relative signal intensity was transformed into $\log_2$ and *z*-score was calculated using the $\log_2$ value. (B) Quantification of asparaptine. (C) Asparaptine secreted to the liquid MS medium from the suspension cells treated with phytohormones. DMSO, dimethylsulfoxide; MeJA, methyl jasmonate; GA_3, gibberellin A_3; IAA, indole acetic acid.

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